Plant Cell Monogr (2)
A. Mujib · J. Šamaj: Somatic Embryogenesis
DOI 10.1007/7089_032/Published online: 9 December 2005

Somatic Embryogenesis of Pine Species: From Functional Genomics to Plantation Forestry

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Abstract Several economically important tree species belong to the genus *Pinus* and many of them form the ecological base of forest ecosystems. Pine wood is an important raw material for the forest industry and many of the pine species have been involved in conventional tree improvement programmes. A lot of effort has been made in the development of vegetative propagation methods, especially somatic embryogenesis, in order to rapidly gain the benefits of traditional breeding to be utilized in reforestation. The economically relevant clonal plantation forestry presumes effective mass-propagation systems with high-quality somatic embryo plants. Today this is feasible only for *Pinus banksiana* Lamb., *P. taeda* L. and *P. radiata* D. Don. The recent progress in somatic embryo production and the challenges in functional genomics have increased the understanding of pine zygotic embryo development, leading to improved protocols for somatic embryogenesis. Therefore, clonal plantation forestry might become a reality for more pine species in the coming years. This chapter highlights the recent challenges in the functional genomics of pine embryogenesis. Possibilities for molecular breeding or utilization of somatic embryo plants in conventional breeding and in clonal plantations in line for sustainable forestry are also covered. The importance of cryopreservation for elite genotype preservation and as a storage method during progeny testing is discussed, as well as the use of ectomycorrhizal fungi during somatic embryo conversion in vitro and acclimatization ex vitro.

1 Introduction

Several economically important tree species belong to the genus *Pinus* of the class Pinaceae and many of them form the ecological base of forest ecosystems. Pine wood is an important raw material for pulp production, saw-timber and the furniture industry. During recent decades the extraction of timber from managed or semi-natural tree plantations, instead of natural woodland, has been considered as sustainable forestry. In Europe these plantations are mainly composed of different species and various genotypes

whereas in America and New Zealand, for example, plantations are composed of single species and a few genotypes. All natural and managed forest areas have a role in forest biodiversity and conservation. To maintain the present forest biodiversity levels all forests should be managed in an ecologically sustainable way. Today, forests are managed for many different purposes including wood production, recreation, ecological and cultural values, and biodiversity, as well as soil and groundwater protection. This brings new challenges to forest management and silviculture. On the other hand, plantation forestry might help to conserve the natural forests especially in developing countries, in which the majority of wood for construction and fires is supplied by natural forests (Walter 2004).

Many of the economically important pine species have been involved in conventional tree improvement programmes. Quite a lot of effort has been put into the development of vegetative propagation methods, especially somatic embryogenesis, in order to rapidly gain the benefits of traditional breeding to be utilized in reforestation. The economically relevant clonal plantation forestry presumes effective mass-propagation systems with high-quality somatic embryo plants. Today this is feasible only for three pine species: *Pinus banksiana* Lamb. (Park 2002), *P. taeda* L. and *P. radiata* D. Don (Smith et al. 1994; Handley et al. 1995; Sutton 2002; Attree 2004). The general obstacles in root production, conversion and acclimatization to ex vitro that hinder any technological outcomes in several pine species could be relieved by inoculation with specific ectomycorrhizal fungi (Niemi et al. 2004). In general, the recent progress in somatic embryo production (e.g. Pullman et al. 2003a) and the challenges in functional genomics have increased our understanding of pine zygotic embryo development, leading to improved protocols for somatic embryogenesis. Therefore, the economically relevant clonal plantation forestry might become a reality for more pine species in the coming years.

Recently, the potential for molecular breeding has also been considered. The classical tree-breeding work in pines is hindered by long life cycles and long generation intervals. Sexual or somatic hybridization may be limited by the sterility of the descents and the genetic barrier between the species. Overcoming this genetic barrier is only possible via genetic transformation. Important future approaches are considered to be the reduction of generation time, production of sterile trees, resistance to pest or fungal diseases and properties of the wood, especially lignin engineering (Peña and Séguin 2001; Diouf 2003). Today, however, the number of stably transformed pine species is limited and the potential practical applications will only be reached in the future. To achieve these goals and/or to apply the technology to conventional tree breeding, it is essential that individual genotypes are conserved during progeny testing in the field. During recent years, cryopreservation protocols have been developed for embryogenic cultures of several pine species.

The present chapter highlights the recent challenges in the functional genomics of pine embryogenesis. The possibilities for molecular breeding, or utilization of somatic embryo plants in conventional breeding or in clonal plantations in line for sustainable forestry, are also covered. The importance of cryopreservation for elite genotype preservation and as a storage method during progeny testing will be discussed, together with the role of ectomycorrhizal fungi within somatic embryo maturation and conversion in vitro and during ex vitro acclimatization.

2 The Present State of the Art in Pine Somatic Embryogenesis

Somatic embryogenesis is a process in which specific somatic cells are genetically reprogrammed towards the embryogenic pathway. Somatic embryo development of pine species encompasses four distinct phases, initiation (Figs. 1a, b), proliferation, maturation (Fig. 1d) and conversion, i.e. germination (Fig. 1e) and subsequent acclimatization ex vitro (Fig. 1g), which are induced by changes of the culture medium composition. After a successful initiation, the embryogenic potential of the proliferating embryogenic mass is maintained on the medium with high concentration of both auxin and cytokinin. Removal of these plant growth regulators is a prerequisite for the development of somatic embryos. During maturation, storage substances are accumulated and somatic embryos differentiate, desiccate and reduce their metabolic activity. These changes are induced by exogenous abscisic acid (ABA) and increased osmolality due to exogenous polyethylene glycol (PEG), sugars or increased gel strength of the medium. For germination, mature somatic embryos are usually cultivated on the medium without exogenous plant growth regulators and with lower concentrations of nutrients and sugar, which induces utilization of storage compounds in embryos. Germination and subsequent root elongation in vitro are critical phases for later acclimatization to ex vitro conditions in a greenhouse.

In pine species immature megagametophytes containing immature zygotic embryos have been the most responsive explants for the initiation of somatic embryogenesis (Handley et al. 1995; Häggman et al. 1999; Percy et al. 2000; Pullman et al. 2003b; Miguell et al. 2004; Niskanen et al. 2004). Somatic embryogenesis from mature zygotic embryos (Tang et al. 2001a; Malabadi et al. 2002) and vegetative shoot apices (Malabadi and van Staden 2005) have also been documented. Likewise, somatic organogenesis from mature zygotic embryos has been regarded as an alternative for somatic embryogenesis in *P. taeda* (Tang and Guo 2001; Tang et al. 2001c; Tang et al. 2004). The whole developmental process from initiation to conversion has succeeded in several pine species, including *P. banksiana* (Park 2002), *P. kesiya* Royle ex. Gord (Malabadi et al. 2002), *P. monticola* Dougl. (Percy et al. 2000), *P. patula* Schede et Deppe

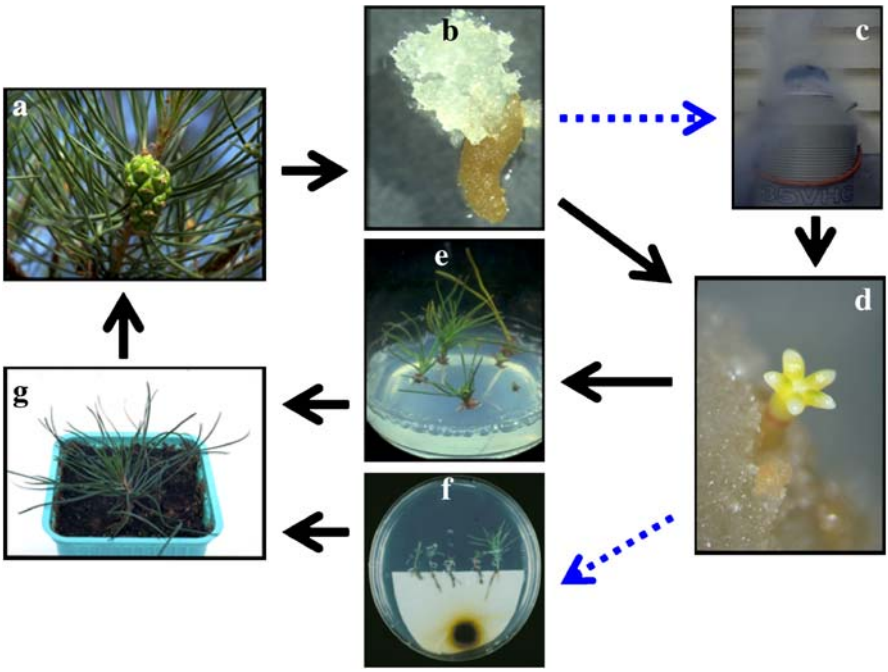

Fig. 1 Somatic embryogenesis of *P. sylvestris*. **a** Developing green cone shortly after meiosis. **b** Initiation of somatic embryogenesis using immature embryos surrounded by megagametophytes and proliferation of embryogenic cell mass. **c** Option for cryopreservation of the germplasm. **d** Maturation of somatic embryos. **e** Conversion of somatic embryo plants. **f** Inoculation with specific ECM fungus improves root development. **g** Acclimatization to ex vitro conditions in a greenhouse

(Jones and van Staden 1995), *P. pinaster* Soland., non Ait. (Lelu et al. 1999; Miguel et al. 2004), *P. radiata* (Sutton 1999; Attree et al. 2004), *P. strobus* L. (Garin et al. 1998; Klimaszewska et al. 2001; Park 2002), *P. sylvestris* L. (Häggman et al. 1999; Lelu et al. 1999) and *P. taeda* (Handley et al. 1995; Sutton 2002; Attree 2004). Recently, a number of selection programmes have been started, predominantly by private forest companies, to test pine embryogenic clones (reviewed by Cyr and Klimaszewska 2002), and for *P. radiata* and *P. taeda* commercial production has dramatically increased (Sutton 2002; Attree et al. 2004).

Recently, somatic embryo production has been improved by achievements in functional genomics and physiology during pine zygotic embryogenesis, as well as by optimization work at the tissue culture media level and during acclimatization. Regardless of the developments, the application of somatic embryogenesis for most pine species is still limited, which is mainly due to low, cell line- and family-dependent initiation frequency and an inability of initiated cultures to become stable during proliferation. Furthermore,

the inability of somatic embryos to fully mature results in low germination frequency and subsequently poor acclimatization ex vitro (Garin et al. 1998; Häggman et al. 1999; Pullman et al. 2003b; Miguell et al. 2004; Niskanen et al. 2004; Malabadi and van Staden 2005).

3 Functional Genomics of Pine Embryogenesis

All pine species have 12 pairs of chromosomes with essentially similar morphology (Sax and Sax 1933). The genome size is large, but there is variation between the pine species (Bogunic et al. 2003). Pine genomes are known to contain highly repeated DNA sequences (Kriebel 1985) and to harbour large complex gene families (Kinlaw and Neale 1997). However, the isozyme profiles of pines show less evidence for large gene families than is apparent from Southern hybridizations (Perry and Furnier 1996). Expressed sequence tag (EST) projects also suggest that the number of expressed gene family members may not be very high. On the other hand, the number of related non-expressed pseudo-genes is higher than in many other plant groups (Komulainen et al. 2003).

Recently, a programme on the functional genomics of *P. taeda* zygotic and somatic embryogenesis has been commenced (Cairney et al. 2003), and the development of a 10 000-clone *P. taeda* cDNA array enriched in sequences expressed in embryogenesis is in progress. Due to the success of heterologous hybridization in conifers (Van Zyl et al. 2002), this microarray will serve as a general pine cDNA allowing high-throughput gene expression analyses. Komulainen et al. (2003) found that the EST-based genetic maps between *P. sylvestris* and *P. taeda* are largely colinear. What is more, a comparative karyotypic analysis of four pine species suggested that the degree of chromosomal differentiation among species is very low (Hizume et al. 2002).

EST microarrays for *P. taeda* have been utilized in several gene expression studies of spruce species (Van Zyl et al. 2002, 2003; Stasolla et al. 2003, 2004). Generally, in somatic embryogenesis of *Picea abies* L. Karst. gene expression is upregulated during transition from proembryogenic masses to embryos, downregulated during early embryogeny and upregulated again at the onset of late embryogeny (Van Zyl et al. 2003). In *Arabidopsis* several regulatory genes responsible for embryo development have been identified by mutant analysis (Jurgens 2001), but in conifers the long generation interval makes the selection of embryo-specific mutants practically impossible. However, genotypes deviating from the normal embryo pattern formation and exhibiting developmental arrest at specific stages represent a tool for studying signalling and gene regulation during embryogenesis in conifers (Van Zyl et al. 2003). In *Picea abies* a comparison between transcript profiles of normal and developmentally arrested embryogenic lines showed that the early phases

of normal embryo development were characterized by a precise pattern of gene expression. Several of these genes encoded proteins that are involved in carbohydrate metabolism, detoxification processes and methionine synthesis and utilization (Stasolla et al. 2004). Stasolla et al. (2003) compared the transcript profiles of stage-specific *Picea glauca* (Moench) Voss somatic embryos matured with or without PEG and found that several genes involved in the formation of the embryo body plan and in the control of the shoot and root apical meristems were up-regulated after PEG treatments. They also observed changes in the transcript levels of the genes involved in sucrose catabolism, nitrogen assimilation and utilization.

Preliminary studies on the molecular mechanisms regulating the phases of pine somatic embryogenesis have revealed several genes with differentially regulated expression between somatic and zygotic embryos. In *P. taeda*, gene expression patterns for 326 differentially expressed cDNA fragments were determined across the sequence of somatic and zygotic embryo development (Cairney 1999). Bishop-Hurley et al. (2003) compared gene expression in embryogenic and non-embryogenic tissues of *P. radiata* and identified six gene families that were preferentially expressed during somatic embryo development in vitro. These gene families include a cytochrome P450 enzyme and four putative extracellular proteins: germin, β-expansin, cellulase and 21-kDA protein precursor.

The understanding of embryogenesis has been increased due to the challenges in functional genomics at the genome, transcriptome and proteome levels. The identified conifer genes that are differentially expressed during embryogenesis are homologous to angiosperm seed storage protein genes (Dong and Dunstan 2000), *lea* genes (Dong and Dunstan 1997), *KNOTTED1*-like homeobox gene (Hjortswang et al. 2002), *HD-GL2* homeobox gene family (Ingouff et al. 2003), *VP1/ABI3* gene family, and $p34^{cdc2}$ protein kinase (Footitt et al. 2003). This suggests that despite the differences in certain aspects of gymnosperm and angiosperm embryogenesis, the genes central to embryogenesis will exhibit a high degree of conservation. Germin-like proteins (GLPs) have also been identified in all plant species examined to date (Khuri et al. 2001). The GLPs have been reported to express in the embryogenic cell cultures of *P. caribea* L. and *P. radiata* (Domon et al. 1995; Bishop-Hurley et al. 2003). Preliminary observations suggest that the gymnosperm GLP *PcGER1* gene is unique in the pine genome (Neutelings et al. 1998), which contrasts with the broad divergence of GLPs among the angiosperms. In our own studies on polyamine biosynthesis in *P. sylvestris* the arginine decarboxylase (*ADC*) gene expression and enzyme activity increased during zygotic embryo development, and the *ADC* mRNA transcripts were localized in specific dividing cells of the shoot meristems of the late embryos (unpublished results). In *P. taeda*, the transcript of an aquaglyceroporin gene, *PtNIP1;1*, was found to be abundant in immature zygotic and somatic embryos, and the gene was expressed preferentially in suspensor tis-

sues (Ciavatta et al. 2001). Ciavatta et al. (2002) suggest that this preferential expression in suspensors was due to specific elements of the putative *PtNIP1;1* promoter.

The rapid increase in the availability of EST sequences has opened new prospects for analysing embryogenesis in conifers. Some pine genes, which are activated or expressed differentially during embryogenesis, have now been isolated. However, the accurate mechanism controlling gene expression and the detailed roles of the genes in directing embryogenesis are not clearly understood. In biological systems, information flow goes from DNA to RNA and further to protein and to metabolites. This means that large-scale protein analyses are needed to complement the data derived from transcriptome analysis. Protein arrays and specific antibodies will be generated and used for the functional characterization of woody plant systems (Cánovas et al. 2004). The precise localization of mRNAs and proteins in embryogenic cells and tissues will provide new insights into the organization of metabolic pathways during pine embryo development. Subsequently, because post-translational factors are functionally important in the cell, metabolome-level studies will be of great importance in gaining a comprehensive view of pine embryogenesis.

4
From Conventional Breeding Towards Molecular Breeding

Generally, vegetative propagation is an important tool for achieving significant credits for both conventional tree breeding and propagation of genetically improved material. By in vitro propagation it is possible to realize additional gain due to the potential exploitation of non-additive genetic variation, to increase homogeneity of the material and to compensate potential shortage of improved seeds from seed orchards. The credits for progeny testing and selection of genotypes for the next generations will also be achieved by testing vegetatively propagated material under various environmental conditions. Somatic embryogenesis is expected to have positive effects on both tree breeding and propagation of conventionally improved pine material. However, for the pine species that have been studied so far, practical applications have been hindered particularly by genotype-dependent initiation, uneven maturation and low germination frequency. Although a lot of prospects have been linked to molecular breeding of coniferous species, problems in both the vegetative propagation and the production of genetically transformed material still limit the biotechnological applications of several pine species. Recent advances in somatic embryogenesis have certainly brought these prospects closer to reality (as reviewed by Merkle and Dean 2000).

The first stably transformed coniferous species, *Larix decidua* Miller, was produced through *Agrobacterium rhizogenes*-mediated genetic transform-

ation (Huang et al. 1991). Since then, *A. rhizogenes* has been considered as a potential tool for rooting recalcitrant woody plants including pine species (as reviewed by Häggman and Aronen 2000). The next stably transformed coniferous species, *Picea glauca*, was achieved by application of particle bombardment technology (Bommineni et al. 1993; Ellis et al. 1993). However, the first report of a stably transformed pine species, *P. radiata*, was published by the New Zealand group of Wagner and co-workers in 1997, which was followed by Walter and co-workers one year later in 1998, i.e. ten years later than the first report on stably transformed hardwood, *Populus alba* × *P. grandidentata* (Fillatti et al. 1987). Later on, both direct gene transfer by particle bombardment, which in most cases means Biolistic® transformation, and *Agrobacterium tumefaciens*-mediated transformation were applied to pine species. *Agrobacterium*-mediated transformation has also been developed as an alternative to Biolistic® transformation for conifers. The advantages of *Agrobacterium*-mediated genetic transformation compared with the Biolistic® method are a lower average copy number, less fragmentation of the transgenes and precise gene integration (Kumar and Faldung 2001, and as reviewed by Walter 2004). Controversially, some papers indicate high integration of vector backbone sequences in plants like rice, tomato, grape and potato after *Agrobacterium*-mediated transformation (Hanson et al. 1999; Kim et al. 2003; Rommens et al. 2004).

At present, regeneration of transgenic pines has been reported via *Agrobacterium*-mediated transformation from organogenic (Tang et al. 2001b) and embryogenic (Tang and Tian 2003) material of *P. taeda*, as well as from embryogenic cultures of *P. strobus* (Levee et al. 1999). In addition to the pioneering work of Wagner et al. (1997) and Walter et al. (1998), transgenic pines via Biolistic® transformation have been produced from embryogenic tissues of *P. radiata* by Bishop-Hurley et al. (2001). Overall, the list of transgenic pines derived from material in tissue culture is still short, only three species. This does not mean that these would be the only pine species which have been targets of genetic transformation, but it might rather reflect the effort put into the development of transformation protocols or severe difficulties in regeneration. As an example, we have studied genetic transformation of organogenic material (Aronen et al. 1994, 1995, 1996; Aronen and Häggman 1995) and embryogenic cultures of *P. sylvestris* (Häggman and Aronen 1998) using both *Agrobacterium*-mediated gene transfer and Biolistic® transformation, but failed to produce transgenic pines mostly due to difficulties in regeneration. In the case of embryogenic cultures, especially slow growth of the cultures together with prolonged antibiotic selection have prevented regeneration. Another example is the work of Wenck et al. (1999), who transformed embryogenic cultures of two coniferous species, *Picea abies* and *Pinus taeda*, using disarmed *Agrobacterium* helper strains to which either a constitutively expressed *virG* or extra copies of *virG* and *virB* were added. Transformation efficiencies in *Picea abies* and *Pi-*

nus taeda increased 1000- and 10-fold, respectively, but regeneration of stably transformed somatic embryo plants was successful only in *Picea abies*.

In species recalcitrant for in vitro propagation, such as *P. sylvestris*, development of protocols without tissue culture would be of great value. One possibility might be to use pollen, which is a natural carrier of genetic material and as such a good target for foreign gene delivery. Transgenic tobacco plants have been regenerated successfully by applying transformed pollen in conventional pollinations (van der Leede-Plegt et al. 1995) or through tissue culture (Stoger et al. 1995). Protocols for the genetic transformation of pine pollen, resulting in transient expression of reporter genes, have been published for *P. banksiana*, *P. contorta* Dougl. ex Loud (Hay et al. 1994), *P. aristata* Engelm., *P. griffithii* McClell, *P. monticola* (Fernando et al. 2000), *P. pinaster* (Martinussen et al. 1995) and *P. sylvestris* (Häggman et al. 1997). For *P. sylvestris* pollen, we developed the particle bombardment protocol and the necessary dehydration–storage protocol for bombarded pollen that is compatible with the conventional crossing technique (Häggman et al. 1997; Aronen et al. 1998). Furthermore, we reported on the production of transgenic plants via the use of transformed pollen in controlled crossings (Aronen et al. 2003). The frequency of transgenic progenies is, however, still low but might be improved by increasing the efficiency of progeny screening. Another option might be to combine the method with the existing somatic embryogenesis protocol for *P. sylvestris*. This means that after controlled pollinations with bombarded pollen, the immature embryos surrounded by the immature megagametophyte could be dissected from the developing cones to initiate somatic embryogenesis.

So far, most of the research on pine species has focused on the development of genetic transformation protocols, and the traits transferred to pine species are listed in Table 1 (reporter genes: β-glucuronidase gene *uidA* or green fluorescent protein gene *gfp*; selectable marker genes: neomycin phosphotransferase *nptII* or hygromycin phosphotransferase *hph*). Considering other traits, there are only two reports that might have feasible options for plantation forestry. Bishop-Hurley et al. (2001) transferred the *bar* gene, which confers herbicide resistance into *P. radiata*, and found that transgenic plants spray tested with Buster (glufosinate) survived with minor or no damage to their needles. Tang and Tian (2003) reported on the integration of the synthetic *Bacillus thuringiensis CRY1Ac* coding sequence, i.e. a modified δ-endotoxin gene to *P. taeda*, and subsequently, in feeding bioassays, they demonstrated an increased resistance to the lepidopteran larvae *Dendrolimus punctatus* Walker and *Crypyothelea formosicola* Staud.

It is clear that the genetic improvement or molecular breeding of all forest crops that utilize genetic transformation techniques is today at an early stage, and forest trees can still be regarded as undomesticated wild trees for the majority of our wood product needs. However, there is a global shift to-

Table 1 The target material, genetic transformation method and transferred genes used in the production of stably transformed pine species

Pinus species	Target material	Transformation method	Gene	Reference
P. radiata	Embryogenic	Biolistic®	*uidA, nptII*	Walter et al. 1998 Wagner et al. 1987
	Embryogenic	Biolistic®	*uidA, nptII, bar, germin*	Bishop-Hurley et al. 2001
P. strobus	Embryogenic	Agrobacterium	*uidA, gfp, nptII*	Levee et al. 1999
P. sylvestris	Pollen	Biolistic®	*uidA*	Aronen et al. 2003
P. taeda	Organogenic	Agrobacterium	*uidA, hph*	Tang et al. 2001b
	Mature, zygotic embryos	Biolistic®	*cry1Ac, nptII*	Tang & Tian 2003

wards tree plantations to meet the increasing need for fibre and to maximize both growth and yield. In this context, the potential of genetically modified tree crops will also be evaluated. At present, the most important approaches include the reduction of generation time, production of sterile trees, resistance to pest or fungal diseases and evaluation of the properties of the wood, especially lignin engineering (Peña and Séguin 2001; Diouf 2003). In addition to these practical goals, a transgenic approach has been widely used as a tool in tree and plant physiology, ecology, genetics and molecular biology (as reviewed by Herschbach and Kopriva 2002). So far, the first and only report in which a transgenic approach has been used to study pine embryogenesis was from Bishop-Hurley et al. (2001), who introduced a specific germin cDNA into *P. radiata* embryogenic cultures.

Biosafety issues of transgenic plants have recently been discussed in several reviews (e.g. Walter 2004) and it has been emphasized, for instance, by the establishment of a Europe-wide, web-based, public-access database (www.versailles.inra.fr/europe/gmorescom) to enhance communication regarding biosafety research. In short, environmental concerns about transgenic technology in plants have arisen from the possibility of not only vertical but also horizontal gene flow, the possible undesirable effects of the transgenes or traits and their possible effect on non-target organisms. All pine species are wind-pollinated, characterized by long life cycles and many of them are the key species of their ecosystems. Therefore, the recognition of the unexpected (e.g. epistatic or pleiotrophic) effects of the transgenes as well as other biosafety concerns have to be considered seriously. However, as also pointed out by Walter (2004), the potential risks or benefits of the genetic modification technology should be discussed in comparison with the risks or benefits of not using this technology.

5
Cryopreservation of Embryogenic Cultures of Pines

Cryopreservation, i.e. storage of material in liquid nitrogen at – 196 °C, represents the only safe and cost-effective option for long-term conservation of plant germplasm (as reviewed by Engelmann 2004). In pine species the recent progress in somatic embryogenesis, the production of genetically modified plants (Table 1) and the efforts towards plantation forestry have emphasized the need for germplasm conservation with functional cryopreservation protocols (Häggman et al. 2000, 2001; Park 2002).

Reliable long-term maintenance of embryogenic cultures requires that the cultures are stored using cryopreservation techniques. It is well known that in conifers the embryogenic cell lines may remain stable for years, the growth and embryogenic potential may vary with time or they may be lost after some months of sub-culturing (as reviewed by Häggman et al. 2000). Recently, it has also been proposed that cryopreservation could be used for cryoselection, i.e. for selection of material with specific properties (Engelmann 2004). In this way it could be used as a tool to "rejuvenate" the cultures with decreasing proliferation capacities (Engelmann 2004), which might be of great value especially for pine species. A protocol for the cryopreservation of conifer embryogenic tissue was first developed by Kartha et al. (1988) and it is still used with minor modifications in conifers including both *Picea* and *Pinus* species. Most of the cryopreservation protocols developed for specific pine species follow the classical cryopreservation techniques that involve the potential pre-treatment of the material and a slow cooling down to a defined pre-freezing temperature, followed by rapid immersion in liquid nitrogen. The material has to be re-warmed fast to avoid the phenomenon of re-crystallization, i.e. re-formation of large and damaging ice crystals by melting ice. This method has been successfully applied with some modifications to several pine species including *P. taeda* (Gupta et al. 1987), *P. caribaea* (Lainé et al. 1992), *P. radiata* (Hargreaves and Smith 1992; Hargreaves et al. 2002), *P. pinaster* (Bercetche and Páques 1995), *P. sylvestris* (Häggman et al. 1998), *P. patula* (Ford et al. 2000) and *P. roxburghii* (Mathur et al. 2003).

New vitrification-based cryopreservation techniques rely on cell dehydration prior to freezing, e.g. by exposure of samples to concentrated cryoprotective medium (Engelmann 1997). Compared with the classical techniques, these new techniques are simpler and have been adopted really quickly for several plant species. At present, in vegetatively propagated species, vitrification-based protocols have been employed almost exclusively (Engelmann 2004). Recently, Touchell et al. (2002) reported in *Picea mariana* the first successful preservation of a coniferous embryogenic culture using a vitrification-based protocol. However, it has not yet been employed with any pine species.

The combining of a clonal forestry strategy with conventional breeding is dependent on cryopreservation. The most important factor in conifer propagation via somatic embryogenesis is the opportunity to cryostore embryogenic lines (Fig. 1c) when the trees are tested in the field. In this way, it is possible to circumvent physiological maturation and hence increase propagation potential. The trees that turn out to be genetically superior in the field may be propagated consistently from the cryogenic storage. Furthermore, as pointed out by Park (2002), sufficient quantities of tested clones can be maintained indefinitely in liquid nitrogen by repeating cycles of cryopreservation, thawing, proliferation and re-cryopreservation. In conclusion, the protocols have to be reliable during the prolonged storage times to ensure genetic stability.

The potential aberrations in genetic stability during cryopreservation might be due to the generally used mutagenic chemical dimethyl sulphoxide (DMSO) in cryoprotectant mixtures (e.g. Häggman et al. 2000), prolonged sub-culturing (DeVerno et al. 1999) and especially in pine species the genetic integrity of clonal lines (Häggman et al. 2000; Park et al. 2002). In pines, megagametophytes may contain multiple archegonia indicating their capability of producing multiple genotypes (e.g. Becwar et al. 1991; Häggman et al. 1998) and the possibility that the subsequently cryopreserved clones may contain mixed genotypes. According to Park et al. (2002), this might be circumvented by re-initiating the cryopreserved clones from mature somatic embryos. This has been achieved from *P. strobus* and *P. banksiana* but at a lower rate (Park et al. 2002). These results emphasize the importance of monitoring the genetic fidelity of cryopreserved material both in vitro and ex vitro at multiple levels. Molecular markers have been used in a few cases. In *Picea glauca*, the genetic stability of randomly selected clones was evaluated by randomly amplified polymorphic DNA (RAPD) fingerprints (De Verno et al. 1999). Variant banding patterns were found in two clones out of six for in vitro culture 12 months after thawing and in plants regenerated from aberrant somatic embryos. De Verno et al. (1999) emphasized the importance of avoiding prolonged sub-culturing as well as the selection of somatic embryos with normal morphology. To our knowledge, the only pine species evaluated by RAPD fingerprints after re-establishment from cryogenic storage is *P. sylvestris* (Häggman et al. 1998), but no variation was found when cryopreserved cultures were compared with unfrozen ones. Overall, molecular markers can be used to detect genetic changes that are not readily expressed as morphological or physiological variations of the phenotype. However, they should preferably be used together with other approaches such as morphological and cytological observations (Fourré et al. 1997). Tsai and Hubscher (2004) pointed out the need to consider additional quality control issues, ranging from the soundness of liquid nitrogen Dewar flasks and cryogenic temperature monitoring to the security of storage facilities and remote backup collections.

6
Obstacles in Conversion and Acclimatization of Pine Somatic Embryos: Do We Need Symbiotic Ectomycorrhizal Fungi?

In nature, all pine species live in mutualistic interaction with specific ectomycorrhizal (ECM) fungi that colonize the roots of the host plant. In ECM symbiosis, the fungal partner increases plant nutrition by increasing the surface that absorbs nutrients and by enabling the use of organic forms of nutrients. Water and nutrients taken up by the fungus are exchanged for carbohydrates derived from the host plant (Smith and Read 1997). To date, genes encoding for nitrate and ammonium transporters have been characterized in an ECM fungus *Hebeloma cylindrosporum* Romagnesi often associated with *P. pinaster* (Jargeat et al. 2003; Javelle et al. 2003), and genes encoding for a general amino acid permease have been characterized in both *H. cylindrosporum* and *Amanita muscaria* (L. ex. Fr.) Pers. (Nehls et al. 1999; Wipf et al. 2002). Furthermore, phosphate, potassium, sulphate and micronutrient transporters were recently identified from a collection of ESTs in *H. cylindrosporum* (Lambilliotte et al. 2004).

The presence of compatible ECM fungi in the pine root system results in dramatic changes in root morphology. Lateral root formation is induced (Tranvan et al. 2000; Niemi et al. 2002, 2005), and furthermore, the tips of short roots may undergo dichotomous branching (Smith and Read 1997). In mature ectomycorrhizas, short roots of the host plant are covered by a hyphal mantle, and a highly branched structure called a Hartig net is formed as the fungus penetrates between epidermal and cortical cells (Smith and Read 1997). The formation of ECM symbiosis causes inhibition in root hair proliferation and external hyphae replace root hairs for absorbing water and nutrients from the soil (Béguiristain and Lapeyrie 1997; Ditengou et al. 2000). The necessity of ECM symbiosis to coniferous species has resulted in attempts to apply ECM fungi in root formation of vegetatively propagated material. Inoculation of the plant cuttings with specific ECM fungi has resulted in a higher rooting frequency, higher number of roots per shoot, and improved root growth of several recalcitrant coniferous species, including pines. However, interaction during root formation has been highly dependent on the plant and fungus genotypes (reviewed by Niemi et al. 2004).

In somatic embryogenesis, successful germination and subsequent growth of the root system are prerequisites for acclimatization to the conditions ex vitro in a greenhouse. However, somatic embryo germination is often poor, and roots elongate and branch slowly or not at all (e.g. Jones and van Staden 1995; Häggman et al. 1999; Niemi and Häggman 2002; Miguel et al. 2004). In nature, germinated seedlings become colonized immediately by mycorrhizal fungi, resulting in better growth of the root system and plant adaptation to the conditions in the soil. Therefore, inoculation with specific ECM fungi might be a potential tool to improve conversion of mature somatic embryos.

So far, there have only been four reports on specific ECM fungi affecting the conversion of mature somatic embryos (Sasa and Krogstrup 1991; Piola et al. 1995; Díez et al. 2000; Niemi and Häggman 2002), one of which is on a pine species (Niemi and Häggman 2002). In our study, four out of five cell lines of *P. sylvestris* increased their germination frequency as a result of inoculation with the ECM fungus *Pisolithus tinctorius* (Pers.) Coker and Couch. Positive responses were observed when the fungal mycelium and germinating embryos were far enough apart to avoid physical contact (Fig. 1f). In contrast, when placed in physical contact, the fungus grew aggressively over the whole embryo. This imbalance between symbiotic partners was probably due to the relatively high concentration of nutrients and sugar in the germination medium. Subsequent inoculation of the germinated somatic embryos with the same fungus on a medium with low nutrient and sugar concentrations resulted in extensive root elongation, root branching and finally mycorrhiza formation (Figs. 2a–c) (Niemi and Häggman 2002). Successful root development and mycorrhiza formation was also observed when somatic embryo plants of *Larix* × *eurolepis* were inoculated with specific ECM fungi (Piola et al. 1995), whereas in *Picea sitchensis* (Bong.) Carr. only a slight or no increase was observed in the growth due to mycorrhiza formation (Sasa and Krogstrop 1991). These results indicate that positive interaction between a somatic embryo and ECM fungus is highly dependent on the developmental phase of the somatic embryo, the fungal and plant genotype, and the composition of the medium.

Similarly, acclimatization of rooted cuttings to the conditions ex vitro was improved in the presence of a specific ECM fungus (Supriyanto and Rohr 1994; Normand et al. 1996). This was also the case with somatic embryo plants

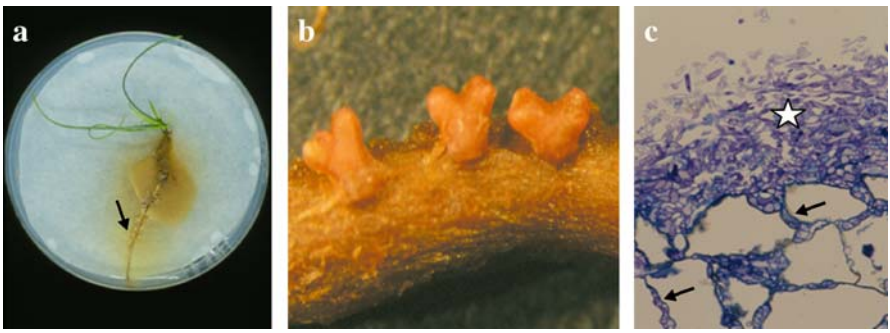

Fig. 2 Ectomycorrhizal symbiosis between *P. sylvestris* somatic embryo plant and *Pisolithus tinctorius* in vitro. **a** An elongated main root of somatic embryo plant and dichotomously branched short roots covered by fungal hyphae (*arrow*). **b** Dichotomously branched mycorrhizal short roots stained red with Ponceau S. **c** Cross section of an ectomycorrhizal short root. Hyphal mantle over the short root (*star*); Hartig net formed by the fungus between epidermal and cortical cells (*arrows*)

of *P. sylvestris* inoculated ex vitro with *Pisolithus tinctorius*. Depending on the plant cell line, better adaptation was observed as either an increased survival rate or increased shoot and root growth. *Pisolithus tinctorius* formed neither hyphal mantle nor Hartig net in the root system, which shows that the plant may benefit from the specific ECM fungus even without mycorrhiza formation (Niemi and Häggman 2002).

Regardless of the necessity for ECM interaction of pines in nature, hardly any attention has been paid to its potential use in somatic embryogenesis. Studies with Scots pine (Niemi and Häggman 2002) and three other tree species (Sasa and Krogstrup 1991; Piola et al. 1995; Díez et al. 2000) clearly show that inoculation with specific ECM fungi is a potential tool for improving both the germination of mature somatic embryos and the acclimatization process of somatic embryo plants. However, since the reactions are highly specific and dependent on the genotypes of both symbiotic partners, it is important to test several fungus strain–plant cell line interactions before any larger-scale use.

7 Concluding Remarks

Pine species are globally important woody species with a wide distribution. During recent decades, plantation forestry has generally been considered as sustainable forestry. Somatic embryogenesis is expected to be a potential mass-scale technology that would allow the production of vegetatively propagated pine clones for reforestation and breeding purposes. Recent achievements in functional genomics, especially in zygotic embryogenesis and physiological outcomes, have improved somatic embryo production. Development of cryopreservation protocols for several pine species have also contributed to practical and tree breeding applications. Nevertheless, there are still obstacles in somatic embryogenesis, e.g. in proper root formation, and certainly more attention should be paid to the potential of natural symbiotic ECM fungi at the germination and acclimatization stages. The progress in somatic embryogenesis has also opened the door to molecular breeding using the transgenic approach. However, this approach is in its infancy and the years to come will show how this technology will be adopted. It is certain that this development has to be based on sustainable forestry.

Acknowledgements We wish to thank Prof. James Graham from the Citrus Research and Education Center, University of Florida, for valuable comments on the manuscript and Mr. Jouko Lehto from the Finnish Forest Research Institute, Punkaharju Research Station, for the photos in Figs. 1 and 2a. We acknowledge the research funding from the Academy of Finland (grants 105214 to H.H., 202415 to K.N. and 53440 to T.S.) and from the Finnish Cultural Foundation (a grant to K.N.).

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